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Journal of Cellular Biochemistry 115:1128–1137 (2014)

Journal of Cellular Biochemistry

Osteoblasts Protect AML Cells From SDF-1-Induced Apoptosis

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ABSTRACT

The bone marrow provides a protective environment for acute myeloid leukemia (AML) cells that often allows leukemic stem cells to survive standard chemotherapeutic regimens. Targeting these leukemic stem cells within the bone marrow is critical for preventing relapse. We recently demonstrated that SDF-1, a chemokine abundant in the bone marrow, induces apoptosis in AML cell lines and in patient samples expressing high levels of its receptor, CXCR4. Here we show that a subset of osteoblast lineage cells within the bone marrow can protect AML cells from undergoing apoptosis in response to the SDF-1 naturally present in that location. In co-culture systems, osteoblasts at various stages of differentiation protected AML cell lines and patient isolates from SDF-1-induced apoptosis. The differentiation of the osteoblast cell lines, MC3T3 and W-20-17, mediated this protection via a cell contact-independent mechanism. In contrast, bone marrow-derived mesenchymal cells, the precursors of osteoblasts, induced apoptosis in AML cells via a CXCR4-dependent mechanism and failed to protect AML cells from exogenously added SDF-1. These results indicate that osteoblasts in the process of differentiation potently inhibit the SDF-1-driven apoptotic pathway of CXCR4-expressing AML cells residing in the bone marrow. Drugs targeting this protective mechanism could potentially provide a new approach to treating AML by enhancing the SDF-1-induced apoptosis of AML cells residing within the bone marrow microenvironment. J. Cell. Biochem. 115: 1128–1137, 2014.

KEY WORDS: AML; CXCR4; SDF-1; CXCL12; OSTEOBLAST; APOPTOSIS; MESENCHYMAL

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by the accumulation of transformed myeloid precursors resulting in impaired production of red blood cells, neutrophils, and platelets as well as, in some cases, diminished immune function. Chemotherapeutics, including cytarabine combined with various anthracyclines, have been the mainstay of AML treatment with most patients experiencing initial remission followed by relapse and death within a few years. Relapse results from the inability of chemotherapeutics to eliminate the AML cells residing in the bone marrow that give rise to further disease [Ishikawa et al., 2007; Konopleva et al., 2009; Doan and Chute, 2012; Emadi and Karp, 2012]. Understanding how to effectively target AML cells within the bone marrow microenvironment is crucial to eliminating the resident AML leukemic stem cells responsible for relapse.

We recently demonstrated that SDF-1 (also known as CXCL12), which is the sole endogenous ligand for the CXCR4 chemokine receptor, robustly induces apoptotic cell death through the intrinsic pathway in AML cell lines and a significant fraction of patient

All authors declare that they have no conflicts of interest.
Grant sponsor: Mayo Foundation; Grant sponsor: Joanne G. and Gary N. Owen Fund in Immunology Research; Grant sponsor: Alma B. Stevenson Endowment Fund for Medical Research; Grant sponsor: National Institutes of Health; Grant numbers: R01 CA166741, U01 CA70095.
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Manuscript Received: 17 December 2013; Manuscript Accepted: 19 December 2013
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 December 2013
DOI 10.1002/jcb.24755 • © 2013 Wiley Periodicals, Inc.

samples expressing CXCR4 on the cell surface [Kremer et al., 2013]. On the other hand, SDF-1 is naturally abundant in the bone marrow microenvironment and is secreted by mesenchymal cells, osteoblasts, endothelial cells, and fibroblasts [Konopleva et al., 2009]. Therefore, SDF-1 should potently induce the apoptosis of AML cells that home to the bone marrow. Nonetheless, AML cells survive and grow in this location. We therefore hypothesized that one or more cell types within the bone marrow microenvironment has the ability to tonically inhibit the SDF-1-driven apoptotic pathway in AML cells.

The endosteal niche, or endosteum, is a protective bone-proximal niche within the bone marrow microenvironment that attracts and supports the survival and maintenance of the pluripotent hematopoietic stem cells (HSC) that give rise to various blood cell lineages. The endosteum is composed of many cell types, including mesenchymal stem cells, stromal cells, osteoprogenitors, osteoblasts, and osteoclasts, but the identity of the cells that protect and nurture HSC and/or AML cells is unclear [Mihara et al., 2003; Levesque et al., 2010; Ehninger and Trumpp, 2011; Singbrant et al., 2011]. Mesenchymal precursors give rise to osteoblasts via a well-characterized developmental program. Osteoprogenitors proliferate and differentiate into osteoblasts that produce extracellular collagenous matrices that become mineralized bone. Osteoblasts continue their differentiation into osteocytes, which reside within the bone and act as mechanosensory cells that regulate osteoblast and osteoclast activity [Jensen et al., 2010; Burgers and Williams, 2013].

No previous studies have addressed the ability of endosteal niche cells to protect leukemic cells from SDF-1-induced apoptosis, however, several studies have described protective features of osteoblast-lineage cells that enhance the ability of primary normal and leukemic haematopoietic cells to survive and grow in vitro in either the presence or absence of chemotherapeutic drugs. Mesenchymal stromal/stem cells can promote AML cell survival via regulation of Bcl-2 [Konopleva et al., 2002] and acute lymphoblastic leukemia (ALL) cell survival via asparagine synthetase [Iwamoto et al., 2007], Notch signaling [Nwabo Kamdje and Krampera, 2011], and Wnt signaling [Yang et al., 2013]. Moreover, mesenchymal stromal/stem cells have been shown to promote the survival of normal HSC cells via mechanisms involving IL-7, SDF-1, stem cell factor, angiopoietin-1, and others [Dazzi et al., 2006; Ehninger and Trumpp, 2011; Cao et al., 2013]. Osteoblasts were previously shown to support HSC survival via distinct mechanisms, including the production of thrombopoietin and secreted frizzle related protein-1 (sfrp-1), and the expression of connexin-43 and Notch ligands [Levesque et al., 2010; Cao et al., 2013].

Like HSCs, AML stem cells home to the endosteal niches [Ishikawa et al., 2007; Doan and Chute, 2012]. Moreover, increased levels of osteoblast-produced osteopontin and thrombopoetin have been linked to worse prognosis in AML patients, suggesting that osteoblasts could mediate the protection of AML cells from chemotherapeutics [Liersch et al., 2012; Dong-Feng et al., 2013]. Thus, osteoblastic lineage cells may provide a safe haven for AML stem cells to survive, not only from chemotherapeutics, but also from the abundant SDF-1 in the bone marrow that could induce their death. Here, we utilized co-culture systems to identify which cells of the osteoblast lineage have the capacity to protect CXCR4-expressing AML cells from SDF-1-induced apoptosis. Our results indicate that differentiating osteoblasts play a crucial role in protecting AML cells from SDF-1-induced cell death in the endosteal niches of the bone marrow.

METHODS

MATERIALS

AMD3100, ascorbic acid, β-glycerol phosphate, hydrocortisone, dexamethasone, and alizarin red were purchased from Sigma–Aldrich (St. Louis, MO). SDF-1 and BMP-2 were obtained from R&D Systems (Minneapolis, MN). A murine monoclonal antibody to CXCR4 conjugated to APC (R&D Systems) was used to assay cell-surface CXCR4 levels on AML cells, and APC-conjugated annexin-V (BD Biosciences, San Jose, CA) was used for flow cytometric assays of AML cell apoptosis.

CELLS

After obtaining informed consent, samples of bone marrow were harvested from AML patients prior to chemotherapy according to an IRB-approved protocol. Following sedimentation on a Ficoll–Paque (1.077 g/cm^3) step gradient [English and Andersen, 1974], mononuclear cells were recovered and cultured in Medium A (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, and 5.5 μM 2-ME) at a density of 1 million cells/ml.

The AML cell lines KG1a and U937 (ATCC, Manassas, VA) were cultured in RPMI supplemented with 10% FCS at a density of 0.6–1.0 million cells/ml. CXCR4-expressing KG1a cells (KG1a-CXCR4) and U937 cells (U937-CXCR4) were generated via transient-transfection of a plasmid encoding a CXCR4-YFP fluorescent fusion protein [Kumar et al., 2006], as previously described [Kremer et al., 2013]. Transfected cells were cultured for 16–18 h to allow expression of the transgene. Transfection efficiencies of KG1a and U937 were typically 10–20% and 10–15%, respectively.

The human bone marrow-derived tert-immortalized BMSC cell line (t-BMSC) was derived from primary mesenchymal cells obtained from unfractionated bone marrow mononuclear cells and transduced with hTERT (a kind gift from Dario Campana, St. Jude, Memphis, TN) [Mihara et al., 2003]. t-BMSC were maintained in RPMI, 10% FCS, 1 μM hydrocortisone, and 2 mM L-glutamine. The liver-derived stromal cell line AFT024 (ATCC) [Moore et al., 1997] was maintained at subconfluence in Medium A.

Primary murine BMSC were flushed from femurs and tibias of 4-week-old C57Bl/6 mice and immediately plated in a 10 cm dish in Medium B (alpha-MEM, 20% FCS, 1% antibiotic/antimycotic, and 1% non-essential amino acids). The following day the cells were plated in 12-well plates at 4×10^6 cells/well in Medium C (α-MEM without ascorbic acid, 20% FCS, 1% antibiotic/antimycotic, and 1% non-essential amino acids). Medium was changed every 2–3 days, and primary BMSC were selected for their ability to adhere to the plate after the first 3 days in culture. Upon reaching 60–70% confluence, the primary BMSC were used in co-culture assays as indicated. All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The Mayo Clinic

Institutional Animal Care and Use Committee approved all animal studies. Animals were housed in an accredited facility under a 12 h light/dark cycle and provided water and food (PicoLab Rodent Diet 20, LabDiet) ad libitum.

MC3T3 sc4 murine calvarial osteoblasts [Wang et al., 1999] (ATCC) were maintained in maintenance Medium D (α-MEM without ascorbic acid (Invitrogen, Carlsbad, VA) containing 10% FCS and 1% penicillin/streptomycin). To induce osteoblast differentiation of MC3T3, cells were plated in 12-well plates. Upon reaching confluence (denoted as Day 0), the medium was replaced with Medium E (α-MEM supplemented with 50 μg/ml ascorbic acid and 4 mM β-glycerophosphate) with medium changes occurring every 2–3 days for the indicated number of days prior to using the cell in co-cultures (see below). Undifferentiated MC3T3 were maintained in Medium D for the indicated number of days starting from confluence in a 12-well plate on Day 0. The differentiation of MC3T3 cells cultured in osteogenic medium was confirmed by fixing the cells with 10% neutral buffered formalin and staining with 2% alizarin red to detect calcified matrix and by quantitative real-time PCR (qRT-PCR) to measure increased expression of the osteoblast marker osteocalcin (as described below).

W-20-17 cells, a kind gift from Vicki Rosen (Harvard School of Dental Medicine, Cambridge, MA) [Thies et al., 1992], were maintained in maintenance Medium F (DMEM, 10% FCS, and 1% penicillin/streptomycin). To induce osteoblast differentiation, W-20-17 cells were plated in 12-well plates and, upon reaching 80–90% confluence (referred to as Day 0), cultured in Medium F with the following osteoblast supplements: 100 ng/ml BMP-2, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Undifferentiated W-20-17 cells were maintained in Medium F for the indicated number of days starting from 80% to 90% confluence in a 12-well plate on Day 0 prior to co-culture (see below). Differentiation was confirmed by measuring gene expression of osteocalcin and alkaline phosphatase using qRT-PCR (see below).

CO-CULTURE ASSAYS AND APOPTOSIS ANALYSIS

The indicated osteoblast lineage cell cultures were washed with PBS prior to addition of 0.25×10^6 cells/ml of either KG1a-CXCR4 or U937-CXCR4 cells in RPMI supplemented with 10% FCS in either the presence or absence of 30 μM AMD3100. Where indicated, cell types were separated by a transwell (Corning, NY; pore size 0.4 μM). The co-cultures were maintained at 37°C for 1 h prior to addition of 1.3×10^{-8} M SDF-1. The co-cultures were then cultured for an additional 16–18 h before AML cell apoptosis was assayed as previously described [Kremer et al., 2013]. Briefly, the apoptosis of transfected cell lines was assayed by staining with APC-conjugated annexin V to detect cell surface phosphatidylserine, subjecting cells to flow cytometry, and using electronic gating to quantitate the binding of annexin V specifically of AML cells expressing similar levels of the transfected CXCR4-YFP. For clinical AML isolates, cells were cultured in Medium A for 1–2 h prior to plating with BMSC and, where indicated, MC3T3. Co-cultures were maintained at 37°C for 1 h, then 5×10^{-8} M SDF-1 was added in Medium A for 16–18 h. Annexin V staining was performed as described [Kremer et al., 2013]. CXCR4 levels were assayed by flow cytometry in clinical AML isolates using a fluorescently conjugated CXCR4 monoclonal antibody after cells were cultured without SDF-1 or stromal cells for 16–18 h [Kumar et al., 2011].

DETECTION OF OSTEOGENIC MARKERS VIA QRT-PCR

RNA was isolated with an RNeasy Plus Kit and reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Gene expression was measured using qRT-PCR. Reactions included 25 ng cDNA per 10 μl with QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and the CFX384 Real-Time System (BioRad, Hercules, CA). Transcript levels were normalized to the housekeeping gene *Gapdh*. Gene expression levels were quantified using the $2^{\Delta\Delta Ct}$ method. Gene specific primer sequences: *Gapdh* (Forward: 5′-GGGAAG CCCATCACCATCTT, Reverse: 5′-GCCTCACC CCATTTG ATGTT), Osteocalcin (*Bglap*) (Forward: 5′-CCTGAGTCTGA-CAAAGCCTTCA, Reverse: 5′-GCCGGAGTCTGTTCACTACCTT), and Alkaline phosphatase (*Alpl*) (Forward: 5′-CACAGATTCCCAAAG-CACCT, Reverse: 5′-GGGATGGAGGAGAGAAGGTC).

RESULTS

MESENCHYMAL STROMAL/STEM CELLS INDUCE DEATH IN AML CELLS EXPRESSING HIGH LEVELS OF CXCR4 AND FAIL TO RESCUE THESE AML CELLS FROM SDF-1-INDUCED APOPTOSIS

We previously demonstrated that SDF-1 induces apoptosis ex vivo in AML cell lines and patient samples that express elevated levels of cell-surface CXCR4 [Kremer et al., 2013]. SDF-1 is abundant in the bone marrow microenvironment, yet AML cells thrive in the bone marrow. This apparent paradox could be explained if a subset of cells within the bone marrow were to protect AML cells from SDF-1-induced apoptosis. To begin to test this hypothesis, we cultured our previously described models of AML cell lines expressing elevated levels of cell-surface CXCR4 [Kremer et al., 2013] together with different cell types found in the bone marrow microenvironment. To mimic primary AML cells from patients, which frequently express elevated cell-surface CXCR4, the human myeloid leukemia line KG1a and the human monoblastic leukemia line U937 cells were transiently transfected with a plasmid encoding CXCR4 as previously described [Kremer et al., 2013]. The presence of a YFP tag fused to the transfected CXCR4 permits the unambigious identification of AML cells that express similar levels of CXCR4 in co-cultures. As previously described [Kremer et al., 2013] (Fig. 1A, left FACS plot), these YFP+ AML cells undergo robust SDF-1-dependent apoptosis (Fig. 1A, left three FACS plots).

Because bone marrow-derived mesenchymal stromal/stem cells (BMSC) can promote the survival of HSCs, ALL, and AML cell lines [Konopleva et al., 2002; Dazzi et al., 2006; Iwamoto et al., 2007; Ehninger and Trumpp, 2011; Nwabo Kamdje and Krampera, 2011; Yang et al., 2013], we examined whether BMSC could also protect CXCR4-expressing AML cells from SDF-1-induced apoptosis. We first utilized t-BMSC, a human tert-immortalized BMSC cell line derived from mesenchymal bone marrow cells [Kumagai et al., 1996; Mihara et al., 2003; Kwong-Lam and Chi-Fung, 2013]. t-BMSC were co-cultured with CXCR4-transfected KG1a cells (KG1a-CXCR4 cells) for 1 h prior to the addition of SDF-1 for an additional 16–18 h. CXCR4-expressing cells were then assayed for apoptosis by

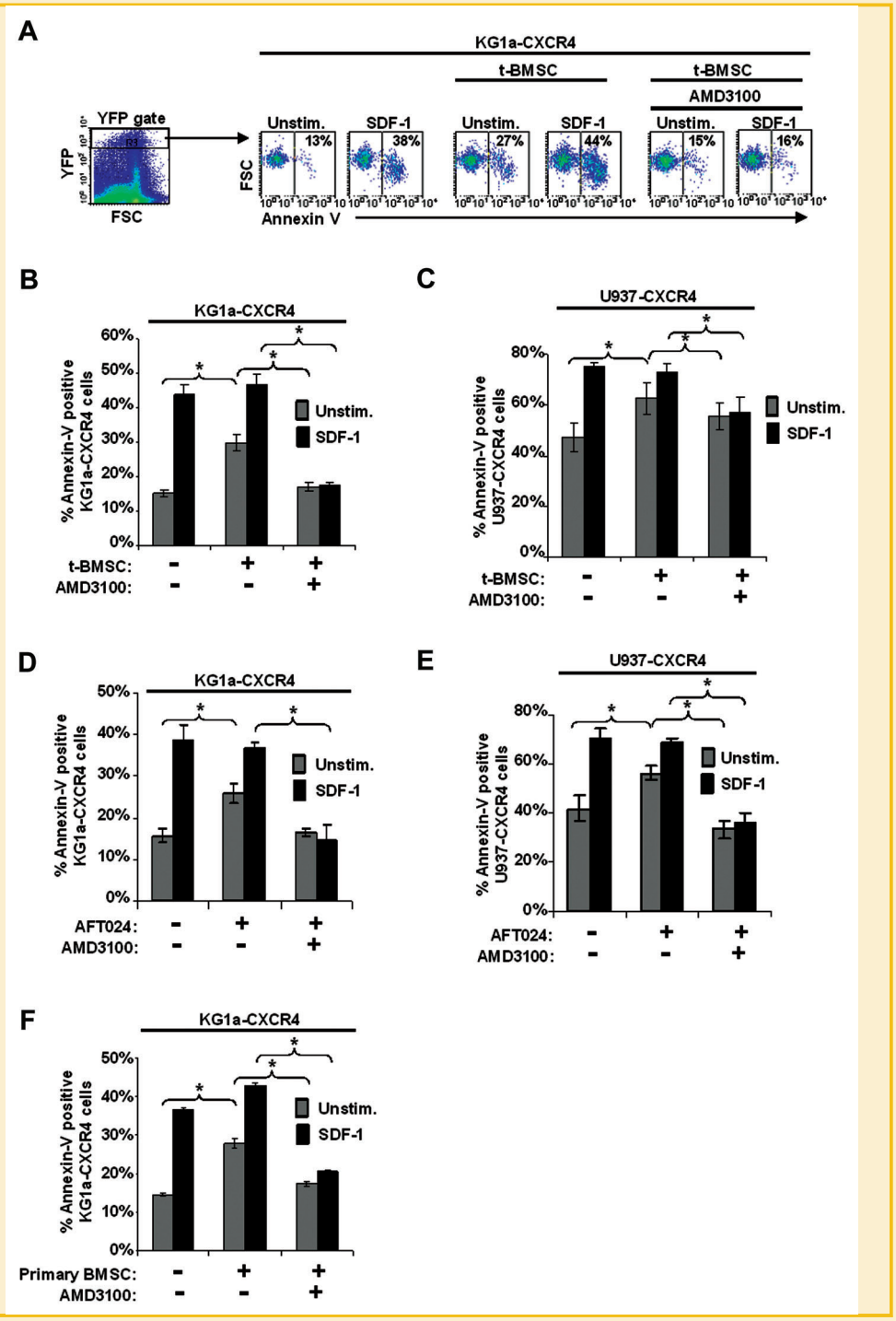


Fig. 1. Mesenchymal stromal/stem cells induce death in AML cells expressing high levels of CXCR4 and fail to rescue these AML cells from SDF-1-induced apoptosis. A,B: Effects of t-BMSC on SDF-1-dependent apoptosis of CXCR4-expressing KG1a AML cells. KG1a cells transfected with CXCR4-YFP were cultured ± freshly plated t-BMSC ± 30 μM AMD3100 for 1 h prior to addition of SDF-1 for an additional 16–18 h, then assayed for apoptosis by staining with APC-conjugated annexin V and analyzing by flow cytometry. Gating as shown was used to measure apoptosis only of KG1a cells expressing similar high levels of CXCR4-YFP (see Methods). In panel A the results of one representative experiment are shown. Panel B summarizes three to six independent experiments performed as in A. Each bar denotes the mean% of YFP+ cells that stained positive for annexin V ± SEM. C: Effects of t-BMSC on the SDF-1-dependent apoptosis of CXCR4-expressing U937 AML cells. Experiments were performed as in panels A and B except that U937-CXCR4 cells were used instead of KG1a-CXCR4. Each bar denotes the mean% of YFP+ cells that were positive for annexin V ± SEM for three independent experiments. D,E: Effects of AFT024 on the SDF-1-dependent apoptosis in CXCR4-expressing KG1a and U937 cells. Experiments were performed as in panels A–C except that AFT024 cells were used instead of t-BMSC. Each bar denotes the mean% of YFP+ cells that were positive for annexin V ± SEM for three independent experiments. F: Effects of primary murine BMSC on the SDF-1-dependent apoptosis in CXCR4-expressing KG1a AML cells. Experiments were performed as in panels A and B except that primary murine BMSC were used instead of t-BMSC. A representative experiment of three is shown. *The results of the indicated conditions were significantly different from each other, $P < 0.05$.

measuring annexin V staining specifically on YFP+ cells. Figure 1A shows data from a representative experiment, while Figure 1B summarizes results of several independent experiments. Interestingly, even when exogenous SDF-1 was not added, coculturing t-BMSC with KG1a-CXCR4 cells resulted in significantly increased KG1a-CXCR4 cell apoptosis ($P < 0.05$, Fig. 1A,B). Because BMSC reportedly secrete SDF-1 [Konopleva et al., 2009], we tested whether the increased apoptosis of the KG1a-CXCR4 cells cultured together with t-BMSC could be blocked by the CXCR4 antagonist drug AMD3100 [Donzella et al., 1998]. Indeed, AMD3100 reduced the percentage of annexin V-positive KG1a-CXCR4 cells in the t-BMSC + KG1a-CXCR4 co-cultures to that of KG1a-CXCR4 cells cultured alone (Fig. 1B). Thus, t-BMSC evidently secrete sufficient SDF-1 to induce CXCR4-dependent KG1a-CXCR4 cell apoptosis. Upon addition of exogenous SDF-1, KG1a-CXCR4 cells further increased their apoptosis despite the presence of t-BMSC (Fig. 1A,B). Similar results were seen when we analyzed a second model AML cell line that we previously showed also undergoes SDF-1/CXCR4-induced apoptosis, CXCR4-transfected U937 cells (U937-CXCR4 cells) [Kremer et al., 2013]. As was the case with KG1a-CXCR4 cells, co-culture with t-BMSC induced the apoptosis of U937-CXCR4 cells in the absence of exogenous SDF-1, and this occurred via a mechanism that was sensitive to AMD3100 (Fig. 1C, gray bars). U937-CXCR4 cells were more susceptible to apoptosis; and adding exogenous SDF-1 did not further increase the apoptosis induced by co-culture with t-BMSCs (Fig. 1C). Thus, co-culture with t-BMSC induced the CXCR4-stimulated apoptosis of AML cell lines, and t-BMSC failed to protect AML cells from apoptosis via this mechanism.

We also analyzed the effects of coculturing AML cells with a second stromal cell line that reportedly supports the survival of stem/progenitor cells, the liver-derived stromal cell line AFT024 [Moore et al., 1997]. Similar to results seen with t-BMSC, coculturing either KG1a-CXCR4 or U937-CXCR4 cells with AFT024 in the absence of exogenous SDF-1 resulted in a significant increase in apoptosis via a mechanism that could be inhibited by AMD3100 ($P < 0.05$, Fig. 1D,E, gray bars). Addition of exogenous SDF-1 failed to further significantly increase the level of apoptosis of either KG1a-CXCR4 cells or U937-CXCR4 cells co-cultured with AFT024 cells, but the AML cell apoptosis was inhibited by AMD3100, indicating that AFT024 induce AML apoptosis by secreting SDF-1 (Fig. 1D,E, black bars).

Finally, we tested whether primary murine bone marrow-derived mesenchymal stromal/stem cells (referred to as "primary BMSC" here and below) can prevent the CXCR4-driven apoptosis of AML cell lines. Similar to results observed with t-BMSC or AFT024 cells, primary BMSC co-cultured with KG1a-CXCR4 cells induced apoptosis of the KG1a-CXCR4 cells in the absence of exogenous SDF-1 via a mechanism sensitive to AMD3100 ($P < 0.05$, Fig. 1F, gray bars). Moreover, coculturing KG1a-CXCR4 with primary BMSC failed to protect the AML cells from apoptosis upon addition of exogenous SDF-1 (Fig. 1F, black bars). Collectively, the results in Figure 1 indicate that BMSC, whether immortalized human or mouse cell lines or primary BMSC, do not protect CXCR4-expressing AML cells from SDF-1-induced apoptosis, but rather are capable of inducing the apoptosis of AML cells in an SDF-1-dependent manner.

DIFFERENTIATING OSTEOBLASTS PROTECT AML CELLS FROM SDF-1-INDUCED APOPTOSIS

Because BMSC did not protect AML cells from SDF-1-induced apoptosis, we analyzed the role of osteoblasts in mediating this protection. Osteoblasts support both normal as well as leukemic hematopoiesis [Bruserud et al., 2004; De Toni et al., 2006; Levesque et al., 2010]. First, we utilized the rapidly mineralizing subclone of the MC3T3 osteoblasts (sc.4) [Wang et al., 1999]. Osteogenic medium was added on Day 0 to induce differentiation of MC3T3 cells. Induction of differentiation was confirmed by both alizarin red staining, an indicator of calcified matrix production by cells of the osteoblastic lineage (Fig. 2A), and production of the osteocalcin transcript as assayed by qRT-PCR (Fig. 2B). For the co-cultures, the osteogenic medium was removed on the indicated day, the MC3T3 cells were washed, the KG1a-CXCR4 cells were added with or without SDF-1, and the co-cultures were maintained for 16–18 h. KG1a-CXCR4 cells, identified by their expression of YFP, were then assayed for apoptosis by flow cytometry as in Figure 1. As shown in Figure 2C, undifferentiated MC3T3 (Day 0) cells failed to protect KG1a-CXCR4 cells from SDF-1-induced apoptosis, but instead substantially increased the apoptosis of the AML cell line in the absence of exogenous SDF-1. In marked contrast, MC3T3 that had been cultured in osteogenic media for 7, 14, or 21 days substantially protected the KG1a-CXCR4 cells from SDF-1-induced apoptosis (Fig. 2C). Since BMSC and osteoblasts coexist in the bone marrow, we also analyzed the effects of combining differentiating MC3T3 with t-BMSC. Although undifferentiated MC3T3 (Day 0) cells failed to substantially protect KG1a-CXCR4 cells from SDF-1-induced apoptosis in the presence of t-BMSC, differentiating MC3T3 were able to protect KG1a-CXCR4 cells from SDF-1-induced apoptosis even in the presence of t-BMSC (Fig. 2D). Together, these results indicate that MC3T3 incubated in differentiation medium for 7–21 days can potently protect CXCR4-expressing AML cells from SDF-1-induced apoptosis in either the presence or absence of BMSC.

DIFFERENTIATING OSTEOBLASTS RAPIDLY ACQUIRE THE ABILITY TO PROTECT AML CELLS FROM SDF-1-INDUCED APOPTOSIS, AND PROTECTION DOES NOT REQUIRE CELL–CELL CONTACT

In further experiments, we asked whether fewer days of osteogenic differentiation would be sufficient to induce this protective ability in MC3T3 cells and we also addressed whether the simple confluency of the MC3T3 cultures over multiple days might by itself provoke the protective effect. Figure 3 shows the results from MC3T3 cells cultured for only 2 days in either osteogenic ("differentiated," Diff) or normal ("undifferentiated," Undiff) culture medium before either KG1a-CXCR4 or U937-CXCR4 cells and/or exogenous SDF-1 were added for 16–18 h. Day 2 undifferentiated MC3T3 cells had modest ability to protect KG1a-CXCR4 cells from SDF-1-induced apoptosis ($P < 0.05$, Fig. 3A). Nevertheless, Day 2 differentiated MC3T3 cells displayed a much greater ability to protect the KG1a-CXCR4 cells, particularly when exogenous SDF-1 was added ($P < 0.05$, Fig. 3A). Moreover, only the Day 2 differentiated MC3T3 were able to significantly protect the other CXCR4-expressing AML cell line (U937-CXCR4 cells) from SDF-1-stimulated apoptosis ($P < 0.05$, Fig. 3B). MC3T3 cells cultured in osteogenic medium for only 1 day were unable protect KG1a-CXCR4 or U937-CXCR4 cells from apoptosis in response to SDF-1

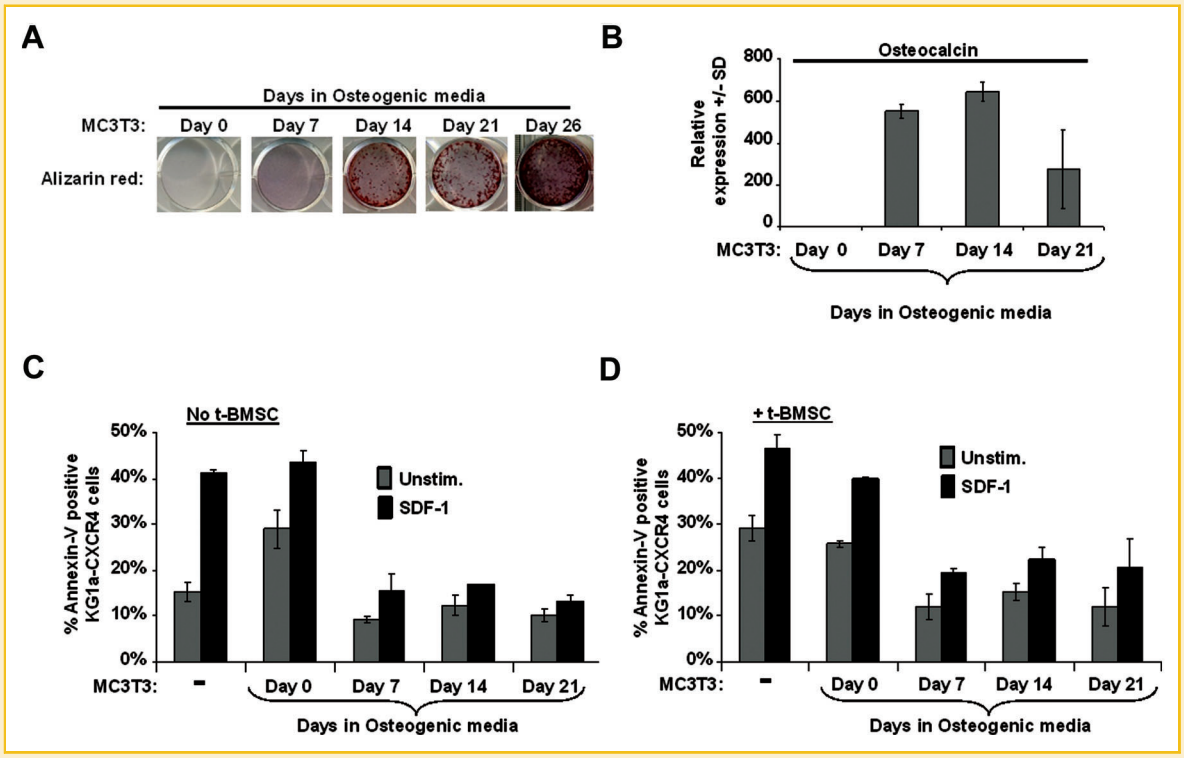


Fig. 2. Differentiating osteoblasts protect AML cells from SDF-1-induced apoptosis. MC3T3 cells were cultured in osteogenic medium for the indicated number of days. A: MC3T3 differentiation was confirmed by alizarin red staining for bone matrix formation at the indicated time points. The results for one representative experiment are shown. B: MC3T3 differentiation was further confirmed by assaying osteocalcin mRNA at the indicated time points via qRT-PCR. The results shown are normalized to the reference gene *Gapdh*. Each bar denotes the mean relative mRNA expression compared to Day 0 ± range for two independent experiments. C,D: After the indicated number of days of incubation of MC3T3 in differentiation medium, KG1a-CXCR4 cells ± t-BMSC were added to washed MC3T3 cultures. Co-cultures were maintained at 37°C for an additional 1 h prior to addition of exogenous SDF-1 where indicated for 16–18 h. Apoptosis of the AML cells was then assayed by annexin V staining and gating on YFP+ cells as in Figure 1A,B. Each bar denotes the mean% of annexin V positive AML cells ± range for two independent experiments.

(data not shown). Thus, differentiating MC3T3 cells rapidly acquire the ability to potently mediate the survival of CXCR4-expressing AML cells in the presence of SDF-1.

To determine whether the osteoblast protection is mediated via a soluble factor or a cell contact-dependent mechanism, we performed assays as in Figure 3A but utilized transwell culture plates to separate the Day 2 differentiated MC3T3 (bottom chamber) from the KG1a-CXCR4 cells (upper chamber). Interestingly, the ability of Day 2 differentiated MC3T3 to protect KG1a-CXCR4 cells from SDF-1-induced apoptosis was retained in the transwell cultures ($P < 0.05$, Fig. 3C). Similar results were found using the U937-CXCR4 cells ($P < 0.05$, Fig. 3D). Thus, Day 2 differentiated MC3T3 protect CXCR4-expressing AML cells from SDF-1-induced apoptosis via a cell contact-independent mechanism.

OSTEOBLASTS PROTECT CXCR4-EXPRESSING AML PATIENT ISOLATES FROM SDF-1-INDUCED APOPTOSIS

We previously showed that SDF-1 induced apoptosis in bone marrow aspirates from a subset of AML patients [Kremer et al., 2013]. In our studies, all of the isolates that responded to SDF-1 by increasing apoptosis also had higher levels of CXCR4 (Fig. 4A,C) [Kremer et al., 2013]. Here, we assessed whether the SDF-1-induced apoptosis in these clinical AML isolates can be inhibited by co-culture with osteoblasts. Patient cells were co-cultured with t-BMSC, plus or minus MC3T3 or exogenously added SDF-1 then assayed for apoptosis. As shown in Figure 4B, the addition of MC3T3 to the co-cultures decreased the level of SDF-1-induced apoptosis to basal levels in all three clinical AML samples analyzed. These results provide further support for the idea that osteoblasts protect CXCR4-expressing AML cells from SDF-1-induced apoptosis.

W-20-17 STROMAL CELLS GROWN IN OSTEOGENIC CONDITIONS PROTECT AML CELLS FROM SDF-1-INDUCED APOPTOSIS

Next, we sought to confirm our findings using another osteoblast cell line. The W-20-17 subclone was derived by limiting dilution from W++ mouse bone marrow stroma and was selected based on ability to express osteoblast characteristics when cultured under osteogenic conditions [Thies et al., 1992]. After 3 days of culture in normal (non-osteogenic) medium, we found that these undifferentiated W-20-17 cells potently provoked the apoptosis of KG1a-CXCR4 cells in co-cultures via a mechanism sensitive to AMD3100 ($P < 0.05$, Fig. 5A, gray bars). Undifferentiated W-20-17 cells were also unable to protect KG1a-CXCR4 cells from apoptosis induced by exogenously added SDF-1 (Fig. 5A, black bars). Thus, like bone marrow stromal cells

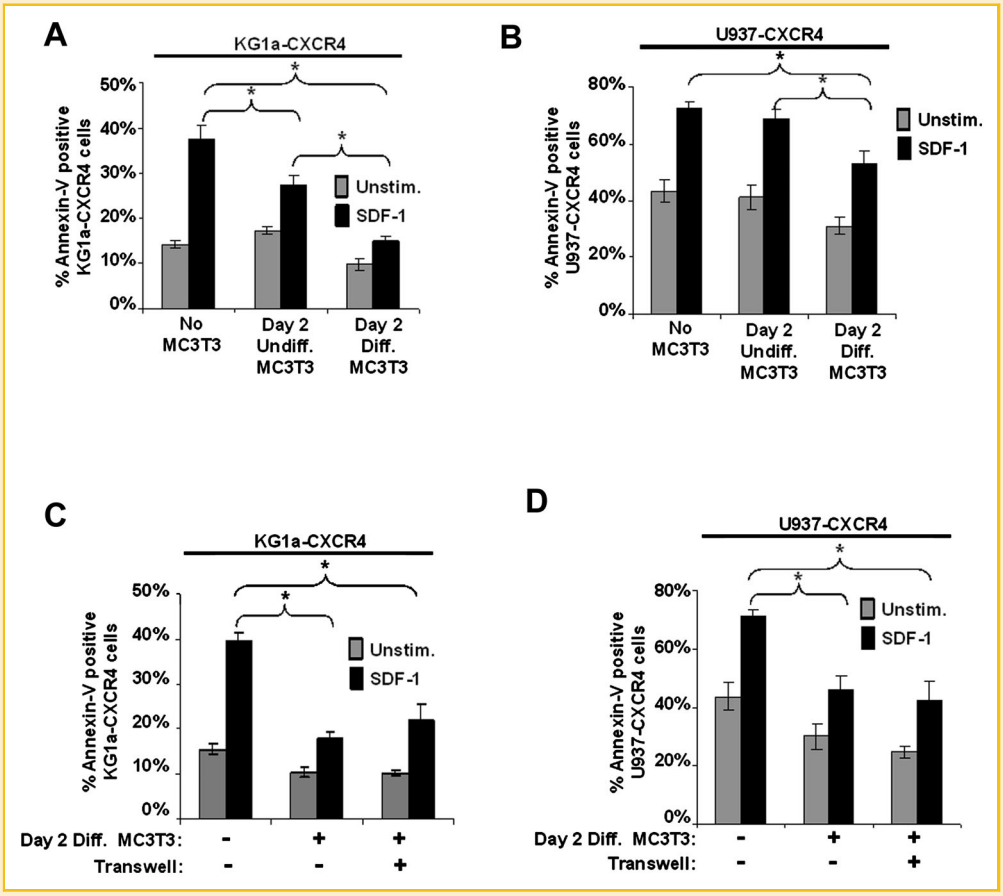


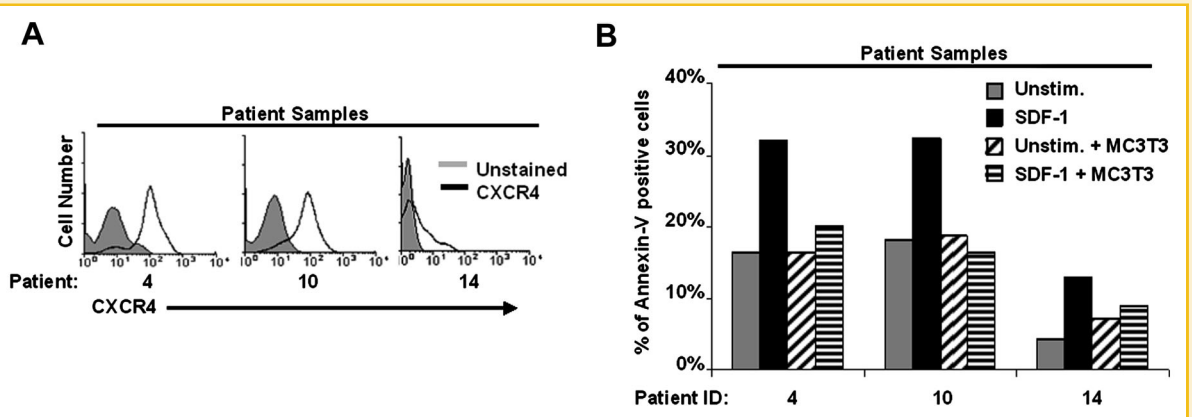
Fig. 3. Differentiating osteoblasts rapidly acquire the ability to protect AML cells from SDF-1-induced apoptosis, and protection does not require cell–cell contact. A,B: MC3T3 cells were grown to confluence (Day 0) and then either maintained as undifferentiated cells or differentiated in osteogenic medium for an additional 2 days. Either KG1a-CXCR4 or U937-CXCR4 cells were then added, co-cultures were maintained at 37°C for 1 h prior to addition of exogenous SDF-1, and then co-cultures were incubated for an additional 16–18 h before the apoptosis of the AML cells was assayed as in Figure 1A–C. Each bar denotes the mean% of YFP-positive AML cells that were annexin V positive ± SEM for three to four independent experiments. C,D: Experiments were performed as in panels A and B except that either the KG1a-CXCR4 cells or the U937-CXCR4 cells were placed in the upper chamber of a transwell system when added to the MC3T3 cells so that co-cultures were performed without direct contact between the AML cells and the MC3T3 cells. Each bar denotes the mean% of YFP-positive AML cells that were annexin V positive ± SEM for three independent experiments. *The results of the indicated conditions were significantly different from each other, $P < 0.05$.

(Fig. 1), undifferentiated W-20-17 cells evidently secrete SDF-1 and thereby induce the CXCR4-driven apoptosis of KG1a-CXCR4 cells instead of protecting these AML cells. In contrast, after 3 days of differentiation in osteogenic medium, W-20-17 cells significantly decreased the extent of SDF-1-induced apoptosis in co-cultured KG1a-CXCR4 cells ($P < 0.05$, Fig. 5B). Differentiation of the W-20-17 cells was confirmed by increased alkaline phosphatase and osteocalcin mRNA expression as assayed by qRT-PCR (Fig. 5C). Together, these results indicate that, like MC3T3 differentiating osteoblasts, differentiating W-20-17 osteoblasts rapidly acquire the ability to protect CXCR4-expressing AML cells from SDF-1-induced apoptosis. Collectively, the results in Figures 1–5 suggest that, within the bone marrow microenvironment, cells at a relatively early stage of osteoblast lineage development are capable of protecting CXCR4-expressing AML cells from SDF-1-induced apoptosis; and these cells are able to protect AML cells despite the presence of other cell types that induce AML cell apoptosis.

DISCUSSION

Endosteal niches are unique environments within the bone marrow that attract and promote the survival of HSC and leukemic stem cells. Endosteal niches provide a distinct physical–chemical environment that promotes the homing and survival of HSC and leukemic stem cells to these niches. In addition, the endosteal niches contain multiple cell types that provide stimuli unique to this region [Konopleva et al., 2009; Levesque et al., 2010; Doan and Chute, 2012]. Identifying the cell types that protect the leukemic stem cells within the endosteal niches may lead to valuable strategies to target either the protective cell type or the signaling pathways that drive the protection in order to eliminate AML cells from the bone marrow.

We showed previously that the chemokine SDF-1, which is secreted by most cell types within endosteal niches, activates the intrinsic pathway of apoptotic death in CXCR4-expressing AML cell



C

Patient	Gender	Age	Karyotype	FAB	Newly dx or relapse	Prior MDS/MPN	Treatment related	FLT3 mutation status[a]
4	Female	61	46 XX	M5	New	No	No	-
10	Female	33	46 XX	M5	New	No	No	+
14	Female	71	46,XX, t(6;11)(q27;q23)	AML, NOS	New	No	No	-

Fig. 4. Osteoblasts protect CXCR4-expressing AML patient isolates from SDF-1-induced apoptosis. A: Bone marrow isolates were harvested from AML patients prior to chemotherapy. Patient samples were cultured for 16–18 h, then levels of CXCR4 protein on the cell surface were analyzed via incubation of intact cells with anti-CXCR4 mAb followed by flow microfluorimetry. Results shown are from three different patients. Samples 4 and 10 were previously utilized to show their SDF-1-dependent apoptosis in the absence of MC3T3 cells as well [Kremer et al., 2013]. B: Patient AML isolates were cultured for 1–2 h prior to being added to t-BMSC ± MC3T3 co-cultures. Beginning 1 h after the cells were combined, co-cultures were treated with 5×10^{-8} M SDF-1 for 16–18 h. Apoptosis of patient AML isolates was assayed via APC-conjugated annexin V staining and flow microfluorimetry. Results shown are from three different patients. C: Patient information. Footnotes: a, + indicates presence of activating mutation (internal tandem duplication or D835 mutations), − indicates absence of activating mutation, N.D. indicates test not done. b, the response of Patients 4 and 10 to SDF-1 in the absence of osteoblasts was previously reported.

lines and patient isolates [Kremer et al., 2013]. Here, we identified a cell type found within the endosteum that is capable of mediating the protection of AML cells from SDF-1-induced apoptosis. As summarized below, precursor mesenchymal stromal/stem cells failed to prevent the SDF-1-induced apoptosis of CXCR4-expressing AML cells. In contrast, we showed that osteoblasts differentiated for just 2 days significantly protect AML cells from SDF-1-induced apoptosis.

To identify endosteum-associated cell types capable of protecting CXCR4-expressing AML cells from SDF-1-induced apoptosis, we utilized in vitro co-culture systems that combined different AML cell lines or clinical AML samples with cells representative of different stages of osteoblast development. The earliest stage we examined was that of mesenchymal stromal/stem cells, the precursors of osteoblasts, which have been reported to promote survival of HSC, ALL, and AML cells in culture and in vivo [Kumagai et al., 1996; Konopleva et al., 2002; Iwamoto et al., 2007; Levesque et al., 2010]. Here, we report that t-BMSC, a human bone marrow-derived mesenchymal stromal/stem cell line that is capable of promoting survival of HSC and ALL cells [Mihara et al., 2003; Iwamoto et al., 2007], was not able to promote survival of either of our previously characterized AML models, KG1a-CXCR4 or U937-CXCR4, in the presence of SDF-1. Rather, t-BMSC induced apoptosis in both AML cell lines in the absence of exogenous SDF-1. The CXCR4 antagonist AMD3100 inhibited the t-BMSC-induced apoptosis of AML cells, indicating that t-BMSC likely induce apoptosis of CXCR4-expressing AML cells via the secretion of SDF-1. These findings were confirmed with AFT024, a liver-derived mesenchymal/stromal cell line, as well as with primary murine BMSC. These results indicate that, unlike CXCR4-expressing AML cells, HSC and ALL cells must either fail to initiate apoptosis in response to SDF-1 or they receive a distinct protective signal from BMSC that overrides the SDF-1-induced apoptosis. Nonetheless, BMSC appear capable of inducing apoptosis in CXCR4-expressing AML cells if the protective mechanism of the endosteal niche could be inhibited.

In contrast to the mesenchymal stromal/stem cells, osteoblasts potently protected AML cell lines and AML patient samples from SDF-1-induced apoptosis. We utilized the osteoprogenitor cell lines MC3T3 and W-20-17, in co-cultures with KG1a-CXCR4, U937-CXCR4, or AML patient cells. These experiments showed that osteoblasts acquire the ability to protect AML cells from SDF-1-induced apoptosis within 2–3 days in osteogenic medium and maintain this protection while still differentiating for at least 21 days.

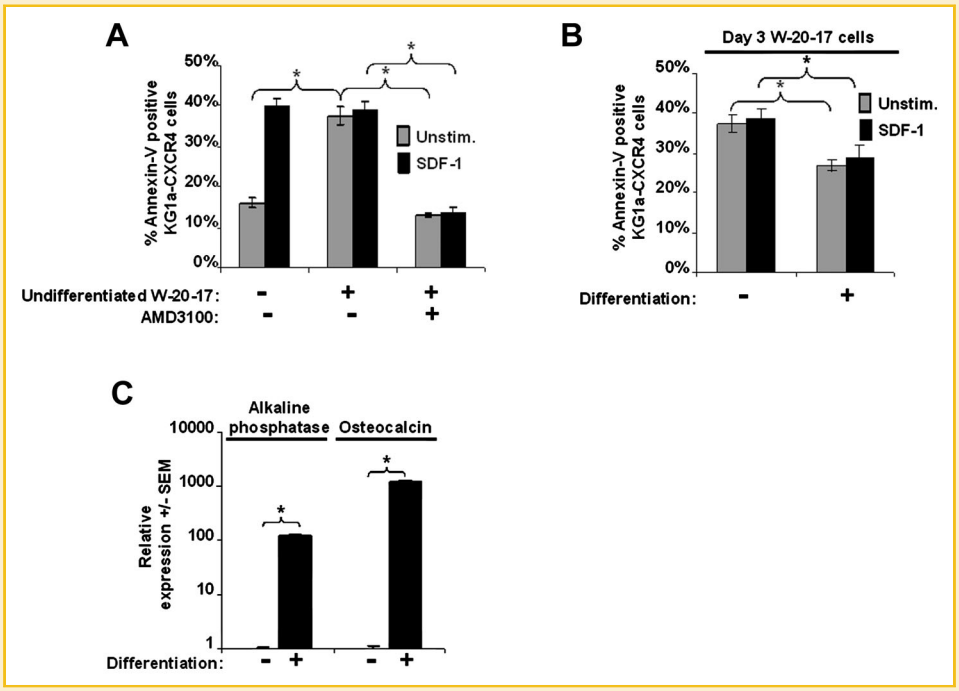


Fig. 5. W-20-17 stromal cells grown in osteogenic conditions protect AML cells from SDF-1-induced apoptosis. A: W-20-17 cells were grown to subconfluence (Day 0) and then undifferentiated cells were cultured for three more days in maintenance Medium F which does not cause their differentiation. On Day 3, W-20-17 cells were washed with PBS prior to addition of KG1a-CXCR4 cells. The co-cultures then were maintained at 37°C for 1 h ±AMD3100, then SDF-1 was added for 16–18 h before the apoptosis of the KG1a-CXCR4 cells was assayed as in Figure 1A,B. Each bar denotes the mean% of YFP-positive cells that were annexin V positive ± SEM for three independent experiments. B: W-20-17 cells were grown to subconfluence (Day 0) and then these undifferentiated cells were cultured for three more days as in A (to remain undifferentiated) while other cells were cultured for three more days in Medium F containing osteogenic supplements to create differentiated W-20-17. On Day 3, the W-20-17 cells were washed with PBS prior to addition of KG1a-CXCR4 cells. The co-cultures were maintained at 37°C for 1 h prior to addition of SDF-1 for 16–18 h. KG1a-CXCR4 cells were assayed for annexin V positive cells as in Figure 1. Each bar denotes the mean% of YFP-positive AML cells that are annexin V positive ± SEM for three independent experiments. C: The mRNA expression of alkaline phosphatase and osteocalcin on Day 3 for the undifferentiated or differentiated W-20-17 cells used in panels A and B was assayed via qRT-PCR and normalized to the reference gene *Gapdh*. Each bar denotes the relative mRNA on compared to Day 3 undifferentiated W-20-17 cells ± SEM, n = 3. *The results of the indicated conditions were significantly different from each other, $P < 0.05$.

Collectively, these results indicate that differentiating osteoblasts acquire a protective phenotype that prevents the SDF-1-induced apoptosis of AML cells cultured either in the presence or absence of apoptosis-inducing BMSC.

Even though the mechanism utilized by osteoblasts to generally enhance survival of HSC and ALL cells cultured in vitro has been extensively studied [Iwamoto et al., 2007; Levesque et al., 2010; Ehninger and Trumpp, 2011], the role of osteoblasts in protecting AML cells has not been well-characterized. De Toni et al. [2006] demonstrated that osteoblasts show a limited protection of U937 cells in the presence of daunurubicin via a mechanism utilizing the Wnt antagonist sFRP-1. Osteoblasts have also been shown to induce the proliferation of a subset of AML blasts without altering viability [Bruserud et al., 2004]. Previously, we demonstrated that a hypoxic environment, such as that found in the endosteum, protects CXCR4-expressing AML cells from SDF-1-induced apoptosis [Kremer et al., 2013]. Here, we used a transwell to show that osteoblasts mediate the protection of AML cells from SDF-1-induced apoptosis via a cell contact-independent mechanism. Although additional studies will be required to determine the precise molecular mechanisms responsible for this osteoblast-mediated protection, several soluble factors involved in enhancing the survival of HSC and ALL cells such as osteopoeitin, sFRP-1, angiopoietin-1, and bone morphogenetic protein-4 [Levesque et al., 2010] could potentially be involved.

Our studies not only identify osteoblasts as key protective cells within the endosteal niche that permit AML cells to survive within the SDF-1-rich environment of the bone marrow, but also begin to identify the specific osteoblast differentiation state required. These results will support the development of future strategies for targeting and killing AML cells residing within this bone marrow niche. For example, drugs targeting the niche's osteoblast-mediated protective mechanism could potentially eliminate AML stem cells in the bone marrow by allowing endogenous SDF-1 to stimulate AML cell apoptosis. Our results additionally suggest that therapies designed to temporarily reduce osteoblast differentiation may be useful as part of induction therapy for AML.

ACKNOWLEDGMENTS

We greatly acknowledge the gifts of reagents from D. Campana, K. Medina, and V. Rosen; assistance of personnel in the Mayo Clinic

Flow Cytometry Core Facility and Johns Hopkins Oncology Center SAC Lab; and the diligence of numerous clinical colleagues in providing the AML specimens used in this study.

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